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Preparation and Optimization of Lipid-Like Nanoparticles for mRNA Delivery

Bin Li and Yizhou Dong

Abstract

Lipid-like nanoparticles (LLNs) have shown great promise for nucleic acid delivery. Recently, we have developed N^1, N^3, N^5 -tris(2-aminoethyl)benzene-1,3,5-tricarboxamide (TT) derived lipid-like compounds, formulated them into TT LLNs for mRNA delivery, and applied an orthogonal array design to facilitate formulation optimization. This chapter focuses on the following contents relevant to lipid-like nanoparticles: formulation method, particle characterization, orthogonal array design, and in vitro assays.

Keywords

Lipid-like nanoparticles (LLNs); Orthogonal array design; Optimization; Delivery efficiency; mRNA delivery

1 Introduction

Lipid-like compounds are termed lipidoids and have been applied to formulate lipid-like nanoparticles (LLNs) [1]. In the past decade, extensive studies have investigated a wide variety of LLNs and their broad applications including delivery of siRNA, plasmid DNA and mRNA, in various cell lines such as endothelial cells, macrophage and tumor cells [1–33]. Some of them possess enormous potential to be developed for treating diverse diseases [27, 28, 33]. Nevertheless, to maximize therapeutic benefits such as efficacy and safety in humans, new LLNs are in great demand. To this end, we have recently designed and synthesized N^1, N^3, N^5 -tris (2-aminoethyl)benzene-1,3,5-tricarboxamide (TT) derived lipid-like compounds [25]. Then, we formulated them into TT LLNs for mRNA delivery based on our previous experiences [16]. LLNs were self-assembled using multiple components including lipid-like compounds, phospholipid, cholesterol, pegylated lipid, as well as the payload, mRNA. In order to maximize the transfection efficiency of this complex system, we employed an orthogonal array design, which was widely used to optimize parameters in the field of chemical engineering (Fig. 1) [34–37]. This approach enabled us to efficiently elucidate the impact of each formulation component and identify the top-performing formulation ratios with significantly reduced experimental volume. Further study indicates optimized LLNs mainly accumulate in the liver and spleen. Moreover, LLNs encapsulated with Factor IX mRNA are capable of producing a therapeutically relevant level of hFIX in both wild-type and FIX-knockout without obvious acute toxicity [25]. In the following

sections, we will describe in details the experimental materials and procedures for preparing and evaluating mRNA-loaded lipid-like nanoparticles.

2 Materials

All buffers and solutions are prepared using nuclease-free water unless otherwise noted.

2.1 Preparation of Nanoparticles

1. Nuclease-free pipette tips and tubes.
2. Ethanol, 200 proof.
3. Lipid-like compounds including but not limited to TT derivatives (synthesized in house according to the ref. 25). Dissolve a certain amount of lipid-like compound in ethanol to get the desired concentration (Typically, 2 mg/mL for in vitro study; 10 mg/mL for in vivo study). Keep at 4 °C.
4. Phospholipids stock solution including but not limited to 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), 2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE) (Avanti Polar Lipids). Dissolve a certain amount of phospholipid in ethanol to get the desired concentration (Typically, 2 mg/mL for in vitro study; 10 mg/mL for in vivo study). Keep at 4 °C.
5. Cholesterol stock solution. Dissolve a certain amount of cholesterol in ethanol to get the desired concentration (Typically, 2 mg/mL for in vitro study; 15 mg/mL for in vivo study). Keep at 4 °C.
6. DMG-PEG₂₀₀₀ stock solution. Dissolve a certain amount of DMG-PEG₂₀₀₀ in ethanol to get the desired concentration (Typically, 0.4 mg/mL for in vitro study; 4 mg/mL for in vivo study). Keep at 4 °C.
7. mRNAs including but not limited to firefly luciferase mRNAs (FLuc mRNA, TriLink Biotechnologies). Store at −80 °C in small aliquots.
8. Nuclease-free water.
9. 10 mM citrate buffer. Mix 41 mL of 10 mM citrate acid with 9 mL of 10 mM trisodium citrate.
10. 0.1 or 0.3 mL syringe.
11. Microfluidic mixer. LLNs used for in vivo study are prepared by fast mixing of formulation components via a microfluidic mixing device produced by Precision NanoSystems.

2.2 Evaluation of Nanoparticles

1. Ultrapure water with resistivity greater or equal 18.2 MΩ cm, obtainable from a water purification system such as the Milli-Q water purification system (Millipore). This is also referred to as Milli-Q water.

2. 96-well plates, tissue-culture treated.
3. 96-well opaque white plates.
4. Eagle's Minimum Essential Medium.
5. Heat-inactivated fetal bovine serum (FBS).
6. 0.05% trypsin–EDTA.
7. Quant-iT RiboGreen RNA reagent (Life Technologies).
8. Bright-Glo luciferase assay substrate (Promega; Store small aliquots at -20°C in darkness).
9. Tris–EDTA solution (10 mM Tris–HCl, 1 mM EDTA).
10. 1% Triton X-100. Transfer 10 μL of Triton X-100 to 990 μL of Tris–EDTA buffer.
11. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) stock solution. Dissolve 0.5 g MTT in 100 mL water to obtain 5 mg/mL solution. Keep at -80°C in small aliquots from light.
12. Dimethyl sulfoxide (DMSO).
13. Hep 3B cells.
14. Particle size and zeta potential analyzer. In this work the NanoZS Zetasizer (Malvern) has been used.
15. Microplate reader for fluorescence intensity detection (wavelength range 200–1000 nm). In this work, the SpectraMax M5 plate reader (Molecular Devices) has been used.
16. Device for automated vitrification. In this work, a Vitrobot Mark IV system (FEI) has been used.
17. Transmission electron microscope. In this work, a Tecnai F20S/TEM (FEI) has been used.
18. Cryo-transfer holder compatible with electron microscope. For this work, a Gatan 626 cryotransfer holder (Gatan) has been used.

3 Methods

All procedures are performed at room temperature unless otherwise noted.

3.1 Preparation of mRNA-Loaded Lipid-Like Nanoparticles

1. To prepare x μL (the final concentration of mRNA is y $\mu\text{g}/\mu\text{L}$) of LLN, dissolve each component (lipid-like compound, phospholipid, cholesterol and DMG-PEG₂₀₀₀) except mRNA separately in 200 proof ethanol in individual RNase-free tubes as stock solutions (*see* Notes 1 and 2).

2. Combine $10xy$ μg of lipid-like compound with phospholipids (*see* Note 3), cholesterol, and DMG-PEG₂₀₀₀ into a 1.5 mL RNase-free tube at molar ratio 50:10:38.5:1.5 according to the ratio from ref. 30.
3. Add $x/40$ μL of 10 mM citrate buffer.
4. Make up to the final desired volume of $x/4$ μL with ethanol. If $x < 1000$ μL and $y < 0.02$ $\mu\text{g}/\mu\text{L}$, proceed with **step 5a, b** of the protocol. Otherwise, proceed with **step 5c–f**.
5. (a) To the above solution, add an equal volume ($x/4$ μL) of mRNA (xy μg) dissolved in 10 mM citrate buffer (pH = 3) by vigorously pipetting the mixture up and down (usually 50–60 times). (b) Add $x/2$ μL of PBS with the same procedure to form homogeneous particles (*see* Notes 4 and 5). (c) Transfer the above solution to a 0.1 mL syringe. (d) Afterward, dissolve xy μg of mRNA in $x/4$ μL of 10 mM citrate buffer (pH = 3). (e) Combine it with twofold volume ($x/2$ μL) of PBS and transfer to a 0.1 mL syringe. (f) Particles are then assembled through a microfluidic-based mixing device following the manufacturer's instructions (*see* Note 6).
6. Use freshly prepared LLNs for subsequent characterization and transfection, and store the remaining formulation at 4 °C (*see* Note 7).

3.2 Characterization of Lipid-Like Nanoparticles

1. Measurement of particle size and zeta potential: disperse 0.3/ y μL of LLNs into Milli-Q water to a final volume of 1 mL in disposable cuvette to make a 300 ng/mL working solution (*see* Note 8).
2. Determine the particle size and zeta potential of LLNs using a NanoZS Zetasizer after mixing well.
3. Measurement of mRNA entrapment efficiency: Prepare a 500 ng/mL working solution in TE buffer as described above.

¹Special attention should be given when working with RNA. Assign specific benchtop and set a dedicated set of pipettes for RNA work. Spray the benchtop with RNase AWAY solution (or equivalent) to eliminate RNase contamination before preparing work solution.

²The concentration of each component should be adjusted according to its molecular weight. For better accuracy, ensure that stock concentration is not too high. Otherwise, the required volume is too small to be accurate; conversely, if the total volume of combined components is more than $x/4$ μL , simply increase the concentration to some extent. Typically, $x = 200$, $y = 0.01$.

³Store phospholipids stock at 4 °C and discard solutions exceeding one week.

⁴To minimize ethanol evaporation, close the tube cap between intervals.

⁵It is not uncommon to produce air bubbles during pipetting. Conversely, in most cases, it is an obvious feature associated with the formation of LLNs. Bubbles will disappear rapidly from solution once mixing is stopped.

⁶If a large-volume and highly concentrated LLNs are required for an in vivo study, use 3 mL syringe. It is necessary to get rid of ethanol by dialyzing samples using 1 L of $1\times$ PBS for 60–90 min at room temperature with dialysis cassettes (3500 MWCO) before injection into animals.

⁷LLNs should be stable for over four weeks at 4 °C since no significant size change is observed during this period. Nevertheless, we recommend using freshly made LLNs.

⁸Prior to measurement, gently invert the tube several times to obtain homogenous formulations. Do not vortex or fiercely agitate the solution, which may destroy the nanostructure of formulations.

4. Add 50 μ L of working solution (use TE buffer as blank) to each well of 96-well plate in triplicate, followed by adding 50 μ L of TE buffer or 1% Triton X-100 (v/v, in TE buffer) (*see* Note 9).
5. To this solution, add 100 μ L of 200-fold diluted Quant-iT RiboGreen RNA reagent (diluted in TE buffer).
6. Incubate for 15 min at 37 °C in the dark.
7. Measure the fluorescence on a SpectraMax M5 plate reader (Molecular Devices) with an excitation wavelength of 480 nm and emission wavelength of 520 nm. The entrapment efficiency is determined as $[1 - (A_{TE} - A_{blank}) / (A_{1\% \text{ Triton}} - A_{blank})] \times 100$.
8. Cryo-TEM: Add approximate 3 μ L of LLNs to a specimen grid.
9. Remove any excess solution.
10. Plunge the grid into liquid ethane using a vitrification device.
11. Transfer under liquid nitrogen to a cryotransfer holder that is loaded to an electron microscope.
12. Record Cryo-TEM images on a postcolumn 1k $\times$ 1k CCD camera at a magnification of 18,500 $\times$ (Fig. 2).

3.3 Evaluation of Cytotoxicity of Lipid-Like Nanoparticles

1. Hep 3B cells are maintained in Eagle's Minimum Essential Medium (EMEM) supplemented with 10% heat-inactivated FBS.
2. Detach cells with 0.05% trypsin when they reach approximately 70% confluence.
3. Seed cells into 96-well plates (150 μ L/well) at a density of 20,000 cells per well (*see* Note 10).
4. Treat cells with 20 μ L of freshly formulated LLNs per well for 6 h after overnight culture.
5. Add 17 μ L of MTT per well for 4 h.
6. Carefully remove the whole medium.
7. Add 150 μ L of DMSO.
8. Dissolve MTT by shaking the plate for about 15 min.
9. Measure the absorbance using a plate reader at 570 nm.

⁹Avoid creating bubbles when adding 1% Triton solution. After adding RiboGreen reagent, cover the plate with foil to avoid light since this reagent is susceptible to photo degradation.

¹⁰Keep equivalent cell confluence for each treatment to ensure reproducibility.

3.4 Evaluation of LLNs-Mediated Transfection

1. Seed and treat Hep3B cells as described above except that 96-well opaque white plates are used.
2. Six hours after treatment, carefully remove the medium containing the formulation.
3. Add 100 μ L of a mixture consisting of 50 μ L of serum-free EMEM and 50 μ L of Bright-Glo (*see* Note 11).
4. Cover the plate with aluminum foil.
5. Wait at least 5 min to allow complete cell lysis.
6. Measure the luminescence values using a plate reader.

3.5 Determination of the Optimal Formulation Parameters

1. Identification of lead lipid-like compound: prepare LLNs as mentioned above using newly synthesized lipid-like compounds.
2. Conduct LLNs-mediated cell transfection assay to select the lead lipid-like compound.
3. Optimization of formulation components ratio: choose a proper orthogonal array table based on the number of formulation components (factors) and assigned molar ratios (levels) (*see* Note 12).
4. Prepare formulation according to the orthogonal table with assigned factors and levels.
5. Conduct LLN-mediated cell transfection assays.
6. Predict the best ratio by analyzing the average luminescence intensity (K_n) of each factor (*see* Note 13).
7. Readjust the intervals according to the impact trend of each component and carry out the second or even more rounds of orthogonal optimization until selecting the optimal formulation (*see* Note 14).
8. Optimization of the ratio for pegylation: regulate the pegylation extent of the above predicted formulation while keeping the other component ratios constant.
9. Measure LLNs-mediated luciferase expression.

¹¹Equilibrate the substrate Bright-Glo to room temperature to fully exert enzyme activity. The desired incubation time is 5–10 min to completely lyse cells and yield high luminescence signals.

¹²For the first round of optimization, large intervals between two adjacent levels are recommended in order to identify the impact trend for each formulation component. In this study, $L_{16}(4)^4$ table (Table 1) is chosen because formulation consists of four components, and each component contains four concentrations (Table 2). For simplification, interaction effects are not considered.

¹³Such a design allows rapidly predicting the top-performing formulation from 256 $(4)^4$ theoretical combinations by testing 16 combinations.

¹⁴Theoretically, more rounds of orthogonal optimization lead to higher potency of the predicted formulation. However, the round of orthogonal optimization depends on demand, cost, statistical difference, stability of formulation and so on.

10. Examine the particle size of LLNs twice weekly (Fig. 3) to identify stable and potent formulation (*see* Note 15).

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References

1. Akinc A, Zumbuehl A, Goldberg M, et al. A combinatorial library of lipid-like materials for delivery of RNAi therapeutics. *Nat Biotechnol.* 2008; 26:561–569. [PubMed: 18438401]
2. Whitehead KA, Langer R, Anderson DG. Knocking down barriers: advances in siRNA delivery. *Nat Rev Drug Discov.* 2009; 8:129–138. [PubMed: 19180106]
3. Cho SW, Goldberg M, Son SM, et al. Lipid-like nanoparticles for small interfering RNA delivery to endothelial cells. *Adv Funct Mater.* 2009; 19:3112–3118. [PubMed: 23393492]
4. Love KT, Mahon KP, Levins CG, et al. Lipid-like materials for low-dose, in vivo gene silencing. *Proc Natl Acad Sci U S A.* 2010; 107:1864–1869. [PubMed: 20080679]
5. Schroeder A, Levins CG, Cortez C, et al. Lipid-based nanotherapeutics for siRNA delivery. *J Intern Med.* 2010; 267:9–21. [PubMed: 20059641]
6. Siegwart DJ, Whitehead KA, Nuhn L, et al. Combinatorial synthesis of chemically diverse core-shell nanoparticles for intracellular delivery. *Proc Natl Acad Sci U S A.* 2011; 108:12996–13001. [PubMed: 21784981]
7. Chen D, Love KT, Chen Y, et al. Rapid discovery of potent siRNA-containing lipid nanoparticles enabled by controlled microfluidic formulation. *J Am Chem Soc.* 2012; 134:6948–6951. [PubMed: 22475086]
8. Whitehead KA, Matthews J, Chang PH, et al. In vitro-in vivo translation of lipid nanoparticles for hepatocellular siRNA delivery. *ACS Nano.* 2012; 6:6922–6929. [PubMed: 22770391]
9. Sun S, Wang M, Knupp SA, et al. Combinatorial library of lipidoids for in vitro DNA delivery. *Bioconjug Chem.* 2012; 23:135–140. [PubMed: 22148515]
10. Jiang S, Eltoukhy AA, Love KT, et al. Lipidoid-coated iron oxide nanoparticles for efficient DNA and siRNA delivery. *Nano Lett.* 2013; 13:1059–1064. [PubMed: 23394319]
11. Alabi CA, Love KT, Sahay G, et al. Multiparametric approach for the evaluation of lipid nanoparticles for siRNA delivery. *Proc Natl Acad Sci U S A.* 2013; 110:12881–12886. [PubMed: 23882076]
12. Zhang Y, Pelet JM, Heller DA, et al. Lipid-modified aminoglycoside derivatives for in vivo siRNA delivery. *Adv Mater.* 2013; 25:4641–4645. [PubMed: 23813808]
13. Sahay G, Querbes W, Alabi C, et al. Efficiency of siRNA delivery by lipid nanoparticles is limited by endocytic recycling. *Nat Biotechnol.* 2013; 31:653–658. [PubMed: 23792629]
14. Kanasty R, Dorkin JR, Vegas A, et al. Delivery materials for siRNA therapeutics. *Nat Mater.* 2013; 12:967–977. [PubMed: 24150415]
15. Bogorad RL, Yin H, Zeigerer A, et al. Nanoparticle-formulated siRNA targeting integrins inhibits hepatocellular carcinoma progression in mice. *Nat Commun.* 2014; 5:3869. [PubMed: 24844798]
16. Dong Y, Love KT, Dorkin JR, et al. Lipopeptide nanoparticles for potent and selective siRNA delivery in rodents and nonhuman primates. *Proc Natl Acad Sci U S A.* 2014; 111:3955–3960. [PubMed: 24516150]
17. Dahlman JE, Barnes C, Khan OF, et al. In vivo endothelial siRNA delivery using polymeric nanoparticles with low molecular weight. *Nat Nanotechnol.* 2014; 9:648–655. [PubMed: 24813696]

¹⁵In general, pegylation hampers delivery efficiency of the formulation but significantly enhance particle stability. Consequently, the extent of pegylation should be regulated to balance delivery efficiency and particle stability, making it suitable for desired applications.

18. Dong Y, Eltoukhy AA, Alabi CA, et al. Lipid-like nanomaterials for simultaneous gene expression and silencing in vivo. *Adv Healthc Mater.* 2014; 3:1392–1397. [PubMed: 24623658]
19. Whitehead KA, Dorkin JR, Vegas AJ, et al. Degradable lipid nanoparticles with predictable in vivo siRNA delivery activity. *Nat Commun.* 2014; 5:4277. [PubMed: 24969323]
20. Ball RL, Knapp CM, Whitehead KA. Lipidoid nanoparticles for siRNA delivery to the intestinal epithelium: in vitro investigations in a Caco-2 model. *PLoS One.* 2015; 10:e0133154. [PubMed: 26192592]
21. Eltoukhy AA, Chen D, Veisheh O, et al. Nucleic acid-mediated intracellular protein delivery by lipid-like nanoparticles. *Biomaterials.* 2014; 35:6454–6461. [PubMed: 24831975]
22. Kauffman KJ, Dorkin JR, Yang JH, et al. Optimization of lipid nanoparticle formulations for mRNA delivery in vivo with fractional factorial and definitive screening designs. *Nano Lett.* 2015; 15:7300–7306. [PubMed: 26469188]
23. Khan OF, Zaia EW, Jhunjhunwala S, et al. Dendrimer-inspired nanomaterials for the in vivo delivery of siRNA to lung vasculature. *Nano Lett.* 2015; 15:3008–3016. [PubMed: 25789998]
24. Yin H, Song CQ, Dorkin JR, et al. Therapeutic genome editing by combined viral and non-viral delivery of CRISPR system components in vivo. *Nat Biotechnol.* 2016; 34:328–333. [PubMed: 26829318]
25. Li B, Luo X, Deng B, et al. An orthogonal array optimization of lipid-like nanoparticles for mRNA delivery in vivo. *Nano Lett.* 2015; 15:8099–8107. [PubMed: 26529392]
26. Li B, Luo X, Deng B, et al. Effects of local structural transformation of lipid-like compounds on delivery of messenger RNA. *Sci Rep.* 2016; 6:22137. [PubMed: 26916931]
27. Leuschner F, Dutta P, Gorbato R, et al. Therapeutic siRNA silencing in inflammatory monocytes in mice. *Nat Biotechnol.* 2011; 29:1005–1010. [PubMed: 21983520]
28. Novobrantseva TI, Borodovsky A, Wong J, et al. Systemic RNAi-mediated gene silencing in nonhuman primate and rodent myeloid cells. *Mol Ther Nucleic Acids.* 2012; 1:e4. [PubMed: 23344621]
29. Dong Y, Dorkin JR, Wang W, et al. Poly (glycoamidoamine) brushes formulated nanomaterials for systemic siRNA and mRNA delivery in vivo. *Nano Lett.* 2016; 16:842–848. [PubMed: 26727632]
30. Jayaraman M, Ansell SM, Mui BL, et al. Maximizing the potency of siRNA lipid nanoparticles for hepatic gene silencing in vivo. *Angew Chem Int Ed Engl.* 2012; 51:8529–8533. [PubMed: 22782619]
31. Wang M, Sun S, Alberti KA, et al. A combinatorial library of unsaturated lipidoids for efficient intracellular gene delivery. *ACS Synth Biol.* 2012; 1:403–407. [PubMed: 23651337]
32. Fenton OS, Kauffman KJ, McClellan RL, et al. Bioinspired alkenyl amino alcohol ionizable lipid materials for highly potent in vivo mRNA delivery. *Adv Mater.* 2016; 28:2939–2943. [PubMed: 26889757]
33. Oberli MA, Reichmuth AM, Dorkin JR, et al. Lipid nanoparticle assisted mRNA delivery for potent cancer immunotherapy. *Nano Lett.* 2017; 17:1326–1335. [PubMed: 28273716]
34. He XJ, Long SA, Zheng MD, et al. Optimization of activated carbon preparation by orthogonal experimental design for electrochemical capacitors. *Sci Adv Mater.* 2010; 2:545–551.
35. Zhan JY, Zheng KY, Zhu KY, et al. Chemical and biological assessment of *Angelicae Sinensis Radix* after processing with wine: an orthogonal array design to reveal the optimized conditions. *J Agric Food Chem.* 2011; 59:6091–6098. [PubMed: 21520895]
36. Meng HN, Zhang ZZ, Zhao FX, et al. Orthogonal optimization design for preparation of Fe₃O₄ nanoparticles via chemical coprecipitation. *Appl Surf Sci.* 2013; 280:679–685.
37. Zhao X, Wu X, Zhou H, et al. Optimization study of the preparation factors for argan oil microcapsule based on hybrid-level orthogonal array design via SPSS modeling. *Pak J Pharm Sci.* 2014; 27:2129–2132. [PubMed: 25410084]

Fig. 1.

A schematic illustration of preparation and optimization of lipid-like nanoparticles for mRNA delivery (reproduced from ref. 25 with permission from (2015) American Chemical Society)

Fig. 2.
A representative Cryo-TEM image of optimized LLNs. Scale bar: 200 nm (reproduced from ref. 25 with permission from (2015) American Chemical Society)

Fig. 3.
The particle stability of LLNs after incorporation of DMG-PEG₂₀₀₀ (reproduced from ref. 25 with permission from (2015) American Chemical Society)

Table 1

Four levels assigned to each formulation component (lipidoid, phospholipid, cholesterol, and DMG-PEG₂₀₀₀)

Levels	Formulation components (molar ratio)			
	Lipidoid	Phospholipid	Cholesterol	DMG-PEG ₂₀₀₀
1	30	1.25	18.5	0.75
2	40	2.5	28.5	1.5*
3	50*	5	38.5*	3
4	60	10*	48.5	6

Asterisk indicates the original molar ratio 50:10:38.5:1.5

Table 2

Orthogonal array table L₁₆ (4⁴) used for optimization of formulation components ratio

Formulation no.	Formulation components (mole ratio)			
	Lipidoid	Phospholipid	Cholesterol	DMG-PEG ₂₀₀₀
1	30	2.5	38.5	3
2	40	10	18.5	1.5
3	50	10	38.5	6
4	60	2.5	18.5	0.75
5	30	5	18.5	6
6	40	1.25	38.5	0.75
7	50	1.25	18.5	3
8	60	5	38.5	1.5
9	30	1.25	48.5	1.5
10	40	5	28.5	3
11	50	5	48.5	0.75
12	60	1.25	28.5	6
13	30	10	28.5	0.75
14	40	2.5	48.5	6
15	50	2.5	28.5	1.5
16	60	10	48.5	3